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REMARKS

Applicant respectfully requests reconsideration of the present application in view of the foregoing amendments and in view of the reasons that follow.

A. Claim Amendments

Claim 16 is to be cancelled, while Claims 2, 9, 10 and 13 were previously canceled. Claims 1, 7, 8, 11, 17 and 18 are currently being amended. Claim 19 is added.

After amending the claims as set forth above, claims 1, 3-8, 11-12, 14-15 and 17-18 are now pending in this application. No new matter is added by these amendments. In particular, the amendments to Claims 7, 8, 11, 17 and 18 are grammatical in nature, to conform the wording of the claims to the language of claims from which each depends. In Claim 1, support for the amendments is found in the Specification at places including the following:

CLAIM	LIMITATION	SUPPORT (REPRESENTATIVE)
1	Invention used to treat defects, disease or damage in the brain.	Original Claim 1.
1	Invention uses a neurotrophin encoding viral expression vector into neurons at up to 10 delivery sites in the brain	Original Claim 1; Specification at paragraph [0017].
19	Delivery site chosen so diffusion of the neurotrophin brings it into contact with other targeted neurons, stimulating growth, sustaining activity, or ameliorating defects, disease or damage therein.	Original Claim 1 and, as to diffusion, Specification at paragraph [0022].

Entry of the proposed amendments to the claims is respectfully requested.

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B. Claim Rejections Under 35 USC Section 112, First Paragraph (Written Description)

Claims 1-8, 11-12 and 14-18, prior to amendment, are rejected for lack of written description support. In particular, the Examiner contends that the limitation "two or more" delivery sites is not supported in the Specification. Although Applicant maintains that such limitation is inherently encompassed within the scope of his disclosure that "one or more" delivery sites be utilized (see, e.g., original Claim 1), and that the disputed phrase need not appear *in haec verba* in the Specification, the phrase has nonetheless been amended for purposes of expedited consideration of the remaining limitations of the claims.

Reconsideration and withdrawal of the written description rejection is therefore respectfully requested.

C. Claim Rejections Under 35 USC Section 112, First Paragraph (Enablement)

Claims 1-8, 11-12 and 14-18, prior to amendment, are rejected for lack of enablement. However, the Office Action confirms that the following embodiments are considered to be enabled:

> "...a method for delivery of a vector comprising a nucleic acid encoding NGF or GDNF, operably linked to a promoter, at two or more sites, not more than about 10 mm apart, to a mammalian brain to stimulate growth or sustain activity of neurons..."
>
> Office Action at page 3, bottom paragraph.

Further, the claims are also considered enabled for "methods of treatment for Alzheimer's disease and Parkinson's disease" (Office Action at page 7, second paragraph).

The enablement rejection is therefore understood by Applicant to be directed only to the following matters:

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1. The claims are rejected for not specifying that the neurotrophin encoded is expressed, resulting in the desired biological responses (Action at page 4, third paragraph). This aspect of the rejection has been addressed by the foregoing amendments to Claim 1. Reconsideration and withdrawal of this aspect of the rejection is therefore respectfully requested.

2. The claims are rejected for not being limited to treatment of Alzheimer's and Parkinson's disease, and for not being limited to delivery of NGF and GDNF. Applicant respectfully disagrees with the Examiner's conclusions in this regard.

Firstly, Applicant maintains his contention that the question of whether delivery of neurotrophins to the brain in the manner taught in the Specification enables one of ordinary skill in the art to practice the invention to ameliorate disease, damage or defects in neuronal populations generally, using neurotrophins other than only NGF and GDNF, has been addressed and resolved by other Examiners in prior, related applications.

For the record, Applicant disagrees with the contention (Action, page 7) that such prior decisions need not be granted full faith and credit so long as a group director signs the Office Action disregarding such prior decisions. To the contrary, decisions of previous Examiners with respect to the same subject matter must not be disturbed unless "there is a *clear error* in the previous action or knowledge of *other* prior art" (MPEP Section 706.04; emphasis added). Such predicate grounds for disregarding the prior decisions of the Office with respect to the same subject matter must be identified in the Office Action (ibid.), and at least a primary examiner must give "great care" to considering such stated grounds before authorizing a rejection based on them (ibid.).

No "clear error" in the Office's previous decisions, nor any "other" prior art not previously considered has been identified in the present Office Action. Therefore, the record does not reflect the requisite considered decision by the Office to disregard its previous decisions with respect to the same subject matter evaluated in related applications based on clear error or new art.

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Secondly, with respect to the substance of the rejection as to the identity of the neurotrophin administered, Applicant submits that the record does not support a conclusion that one of ordinary skill in the art of gene therapy of the brain (a population of highly skilled artisans) could not identify a neurotrophin and its corresponding neurotrophin-receptive receptors (as required by Claim 1) in the brain and deliver the former to the latter in accord with Claim 1. To the contrary, the Specification clearly directs the artisan to direct the neurotrophic compositions of the invention to regions of the brain which contain neurotrophin-responsive neurons into adulthood (see Paragraph [0014]), which include, in the brain, the cholinergic basal forebrain neurons, locus coeruleus neurons, entorhinal (cortical) neurons, and thalamic neurons.

The identity and location of such neurons is well known to those of ordinary skill in the art. The invention pertains to a method for delivering neurotrophins to neurons in the brain that are responsive to them, in the sense that the neuron responds by growing or displaying activity. The activity of neurotrophins such as those described at page 7, line 14 through page 8, line 4 of the specification, as well as the identity of neurons responsive to each in the brain, is and was well-known to the art.

For example, see, e.g., Reference A, neurotrophins useful in stimulating tropic or trophic neuronal responses in the brain include NGF, NT-3, NT-4/5, CNTF, BDNF and others (U.S. Patent No. 5,762,926, at Col. 12, lines 44-50 and Col. 12, line 65 through Col. 13, line 10); Reference A33 ((NGF stimulation of forebrain neuronal growth in monkeys); Reference A35 (BDNF maintains activity among dopaminergic neurons in the brain); Reference A42 (NGF responsive neuronal loss in Alzheimer's Disease); Reference A43 (NGF responsive neuronal loss in Parkinson's Disease); Lapchak, *et al.*, Brain Res., 777:153-160, 1997 (of record), single adenovirally delivered GDNF stimulates dopamine release; and, Yan, et al., Exp.Neurol., 127:23-26, 1994 (of record), the distribution of receptors for NGF, NT-3 and BDNF in the forebrain and striatum is known. The common identifying characteristic of relevance for these neurotrophins is that each exerts a trophic or tropic influence on neurons in the mammalian brain, a fact disclosed in the specification (and otherwise well-known in the art).

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Based on the foregoing, it is respectfully submitted that there is no demonstrated reason of record why the present claims could not be practiced with neurotrophins other than NGF and GDNF; or, conversely, why the claims should be limited to practice only with NGF or GDNF when those of ordinary skill in the art can, by following the teachings of the Specification, utilize other neurotrophins described therein.

Consistent with that conclusion, the Examiner's attention is drawn to other claims allowed to Applicant in commonly owned patents directed to practice of the general method of the invention using *any* neurotrophin: U.S. Patent No. 6,683,058 (any neurotrophin delivered using any vector to cholinergic neurons); U.S. Patent No. 6,815,431 (any neurotrophin delivered using any vector to the dopaminergic neurons); U.S. Patent Publication 2001/0043920, now allowed (NGF or GDNF delivered to cholinergic or dopaminergic neurons using any vector). U.S. Patent Publication 2003/0124095 also teaches use of BDNF and NT-4-5 in the cortices by direct delivery of any vector coding for same.

Applicant therefore respectfully submits that the record and art amply support the conclusion that those of ordinary skill in the art can follow the inventor's teachings to use the invention with neurotrophins other than NGF and GDNF (e.g., those described in the Specification at paragraph [0027]) and with a variety of vectors (e.g., those described in the Specification at paragraphs [0033] – [0035]). Reconsideration and withdrawal of this aspect of the enablement rejection is therefore requested.

3. Remaining is the question of whether the invention can be applied to treatment of conditions other than Alzheimer's (AD) and Parkinson's (PD) disease. Applicant submits that the answer is clearly yes.

The biological responses to practice of the invention achieved in the brain include stimulation and growth of affected neurons. Those of ordinary skill in the art will immediately

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appreciate that such stimulation and growth is of benefit not only in reversing the cognitive and functional deficits associated with diseases such as PD and AD, but also in addressing the loss of neuronal function experienced through the normal processes of aging, or through injury, or through other neurodegenerative conditions, such as Huntington's disease. Whatever the cause of neuronal dysfunction in the brain, the effect of practicing the invention are essentially the same: neuronal activity and growth are stimulated to address the dysfunction. The clinically successful protocol provided by the invention therefore has potentially vast applicability beyond the immediate concerns being addressed by its clinical practice in treating AD and PD.

For example, the data provided in Examples II, V and IV hereof demonstrate *in vivo* responses in rats and primates to expressed neurotrophin delivered either directly or indirectly. In combination, these experiments, together with those in non-human primates and humans described in Dr. Tuszynski's Declaration already of record, demonstrate that cholinergic and dopaminergic neuronal deficits can be reversed according to the invention. Although the data generated in the rat experiments were based on modeling AD, the primate data in the Specification was generated in aged animals with neuronal deficits that occur as part of the natural aging process, which include deficits that also occur in AD; e.g., reductions in cholinergic neuronal density, reduced p75 expression, and related cognitive impairments.

Neurotrophin expression by a vector (either direct or one delivered in a host cell) was achieved in both contexts, expression which can ameliorate the neuronal dysfunction. Thus, the stimulation of neuronal growth and activity achieved was effective, irrespective of the context in which the loss of growth or activity occurred.

Further, the inventor has recently reported that treatment of neuronal populations by direct delivery of neurotrophin to the brain according to the invention has promising results for treatment of Huntington's disease as well (see, McBride, *et al., PNAS*, 103:9345 – 9350, June 13, 2006, copy enclosed).

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Cholinergic and dopaminergic neuronal populations treated according to the experiments referenced above are not the only potential beneficiaries of practice of the invention. For example, in co-pending U.S. Published Patent Application No. 2003/0124095, data is presented demonstrating that BDNF delivery to the cortices in aged animals increased BDNF receptor expression lost to the aging process (Example IV, paragraphs [0058] – [0063]) and demonstrably improved the performance of treated animals on cognitive tests, compared to controls (Example II; paragraphs [0044] – [0051]).

Thus, it has been amply demonstrated that those of ordinary skill in the art would understand the Specification to enable their practice of a method for directly delivering a neurotrophin encoding transgene viral expression vector into neurotrophin-receptive neurons at one or more delivery sites in the mammalian brain no more than about 10 mm from another delivery site stimulates growth, sustains activity, or ameliorates defects, disease or damage, in said targeted neurons, as claimed. Reconsideration and withdrawal of the claims rejection under Section 112, first paragraph, is therefore respectfully requested.

C. Response to Rejection of Claims 1-8, 11-12 and 14-18 under 35 USC Section 112, Second Paragraph.

Claim 1 and its dependent claims are rejected as indefinite for lacking a recitation of the result achieved by practice of the invention. Claim 1 has now been amended to provide such a recitation. Reconsideration and withdrawal of the rejection is therefore respectfully requested.

D. Response to Double Patenting Rejection.

In view of the terminal disclaimer over U.S. Patent No. 6,683,058, Applicant understands the Office Action at page 9 to confirm that the double patenting rejection based thereon has been withdrawn.

In view of the terminal disclaimer over U.S. Patent No. 6,451,306, Applicant understands the Office Action at page 10 to confirm that the double patenting rejection based thereon has been withdrawn.

Claims 1, 2, 5, 6, 11, 12 and 18 are rejected on the basis of non-statutory obviousness-type double patenting over claims 1-6, 8 and 10 of U.S. Patent No. 6,815,451. Claim 2 having been previously cancelled, Applicant interprets the rejection as applying to Claims 1, 5, 6, 11, 12 and 18. In that respect, a terminal disclaimer over the '451 Patent is provided herein. Reconsideration and withdrawal of the double patenting rejection based thereon is therefore respectfully requested.

E. Response to Rejection under 35 USC Section 102.

Claims 1 and 2 are rejected as anticipated by Martinez-Serrano, et al. Claim 2 having been previously cancelled, Applicant interprets the rejection as applying to Claim 1.

The Examiner argues that Martinez-Serrano's teaching of delivery of an MMLV retrovirus encoding NGF by engrafting a neural progenitor cell line transfected therewith teaches all the limitations of Claim 1 (Office Action, page 11). In this respect, Applicant notes that the claims are directed to direct (*in vivo*) delivery of a neurotrophin-encoding transgene, not indirect (*ex vivo*) delivery of transfected donor cells. Nothing in the reference teaches *in vivo* delivery of a neurotrophin-encoding vector.

In this respect, Applicant has previously argued that the reference does not teach direct delivery of a growth factor to the brain. The Examiner has questioned the argument, indicating his belief that the invention teaches delivery of a transgene, not a growth factor (protein), and that a growth factor and a neurotrophin (as recited in the claims) are not the same molecule.

To clarify, the invention does provide means to "deliver a therapeutic neurotrophin" to the brain, via the administration route of applying an expression vector that encodes such neurotrophin to the brain, where it is then expressed according to the invention. Thus, although the transgene is the vehicle utilized for delivery, the molecule ultimately delivered is the neurotrophin. Further, the term "neurotrophin" encompasses "growth factor," the root "trophin" meaning growth. Thus, NGF (nerve growth factor) is properly considered a neurotrophin

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(synonym: neurotrophic factor), and is described as such in the Specification (see, e.g., paragraphs [0013] and [0014]).

Terminology aside, it is clear that the reference describes grafting of donor cells, not direct delivery of a recombinant expression vector encoding a neurotrophin as claimed. Thus, for at least this reason, the reference does not anticipate all of the limitations of the claims.

As to Applicant's argument that the reference does not teach the result achieved by the invention, it is contended at page 12 of the Office Action that the result is not a part of the claim, as it is recited only in the preamble. Applicant responds that the result is now recited as part of the claim (the invention "stimulates growth, sustains activity, or ameliorates defects, disease or damage"). No therapeutic result, much less those achievable by the invention, is taught by the reference. Therefore, for at least this reason as well, the reference does not anticipate all of the limitations of the claims.

F. Response to Rejection under 35 USC Section 103.

Claims 1, 2, 3, 8, 11, 12, 14 and 18 are rejected under Section 103 over Lapchak, *et al.* in view of Martinez-Serrano, *et al.*. Claim 2 having been previously canceled, the rejection is considered as applied to Claims 1, 3, 8, 11, 12, 14 and 18.

Lapchak, *et al.* is cited for its description of an experiment in which a single injection of adenovirus encoding GDNF was introduced into rats. The rats had been treated to display a single motor dysfunction associated with Parkinson's disease. As the Office Action notes, the reference fails to provide any guidance regarding the present, multiple injection clinical protocol.

Martinez-Serrano, *et al.* also fails to suggest the invention, for the reasons discussed above. Therefore, even if one were to consider the authors' discussion regarding use of a cell line in conjuction with Lapchak, *et al.*'s suggestion for single injection of a viral vector, one would still not be provided with any guidance sufficient to arrive at the presently claimed invention.

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For at least these reasons, Applicant respectfully submits that the claimed invention is not obvious in view of the cited references. Reconsideration and withdrawal of the Section 103 rejection is therefore requested.

CONCLUSION

Applicant believes that the present application is now in condition for allowance. Favorable reconsideration of the application as amended is respectfully requested.

The Examiner is invited to contact the undersigned by telephone if it is felt that a telephone interview would advance the prosecution of the present application.

The Commissioner is hereby authorized to charge any additional fees which may be required regarding this application under 37 C.F.R. §§ 1.16-1.17, or credit any overpayment, to Deposit Account No. 50-0872. Should no proper payment be enclosed herewith, as by a check or credit card payment form being in the wrong amount, unsigned, post-dated, otherwise improper or informal or even entirely missing, the Commissioner is authorized to charge the unpaid amount to Deposit Account No. 50-0872. If any extensions of time are needed for timely acceptance of papers submitted herewith, Applicant hereby petitions for such extension under 37 C.F.R. §1.136 and authorizes payment of any such extensions fees to Deposit Account No. 50-0872.

Respectfully submitted,

Date 10-16-2006

By Stacy L. Taylor

FOLEY & LARDNER LLP
Customer Number: 30542
Telephone: (858) 847-6720
Facsimile: (858) 792-6773

Stacy L. Taylor
Attorney for Applicant
Registration No. 34,842

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PNAS

Viral delivery of glial cell line-derived neurotrophic factor improves behavior and protects striatal neurons in a mouse model of Huntington's disease

Jodi L. McBride*, Shilpa Ramaswamy*, Mehdi Gasmi†, Raymond T. Bartus†, Christopher D. Herzog†, Eugene P. Brandon†, Lili Zhou*, Mark R. Pitzer‡, Elizabeth M. Berry-Kravis*, and Jeffrey H. Kordower*§

*Department of Neurological Sciences, Rush University Medical Center, 1735 West Harrison Street, Suite 300, Chicago, IL 60612; †Ceregene Inc., 9381 Judicial Drive, Suite 130, San Diego, CA 92121; and ‡Department of Psychology, Grinnell College, 1116 Eighth Avenue, Grinnell, IA 50112

Edited by William T. Greenough, University of Illinois at Urbana–Champaign, Urbana, IL, and approved May 2, 2006 (received for review October 11, 2005)

Huntington's disease (HD) is a fatal, genetic, neurological disorder resulting from a trinucleotide repeat expansion in the gene that encodes for the protein huntingtin. These excessive repeats confer a toxic gain of function on huntingtin, which leads to the degeneration of striatal and cortical neurons and a devastating motor, cognitive, and psychological disorder. Trophic factor administration has emerged as a compelling potential therapy for a variety of neurodegenerative disorders, including HD. We previously demonstrated that viral delivery of glial cell line-derived neurotrophic factor (GDNF) provides structural and functional neuroprotection in a rat neurotoxin model of HD. In this report we demonstrate that viral delivery of GDNF into the striatum of presymptomatic mice ameliorates behavioral deficits on the accelerating rotorod and hind limb clasping tests in transgenic HD mice. Behavioral neuroprotection was associated with anatomical preservation of the number and size of striatal neurons from cell death and cell atrophy. Additionally, GDNF-treated mice had a lower percentage of neurons containing mutant huntingtin-stained inclusion bodies, a hallmark of HD pathology. These data further support the concept that viral vector delivery of GDNF may be a viable treatment for patients suffering from HD.

gene therapy | neurodegeneration | neuroprotection | polyglutamine | adenoassociated virus

Huntington's disease (HD) is an autosomal dominant neurodegenerative disorder resulting from an expanded trinucleotide (CAG: cytosine, adenine, and guanine) repeat at the IT15 locus on chromosome 4 (1) within the huntingtin gene. The abnormal DNA is translated into mutant huntingtin with an expanded glutamine stretch at the N terminus of the protein. The excessive number of glutamine repeats is responsible for the misfolding of huntingtin and the subsequent formation of neuronal inclusions, degeneration of striatal and cortical neurons, and a triad of symptoms including severe motor, cognitive, and psychological disturbances that are ultimately fatal.

To date, HD remains incurable. Several therapies have yielded positive results in animal models, including those that alleviate potential glutamate-induced excitotoxicity such as riluzole and remacemide (2–4); those that increase the production of energy in the form of ATP in the cell, including creatine and coenzyme Q_{10} (5–9); those that inhibit caspase activation and apoptosis, such as minocycline (10, 11); and those that aim at replacing degenerating cells by means of fetal tissue transplantation (12–18). However, when tested clinically, none has made a major impact in the symptomatic treatment of HD, nor have any demonstrated the ability to alter the natural history of the disease by preventing cell death.

Genetic testing can identify mutated gene carriers destined to suffer from HD. Unlike other neurodegenerative disorders, identification of the genetic marker provides the unique opportunity to intercede therapeutically before the onset of symptoms that result from neuronal degeneration. Toward this end, trophic factors in general, and glial cell line-derived neurotrophic factor (GDNF) in particular, have shown promise in animal models of several different neurodegenerative disorders, including HD (19–22). In addition to its potent trophic effects on dopaminergic midbrain neurons (23, 24), GDNF has also been shown to protect striatal, medium-sized spiny GABA projection neurons, the neuronal population most vulnerable in HD (22). Moreover, the expression of GDNF's receptors (GFRα-1 and Ret) is upregulated in striatal neurons and astrocytes after injury (25), supporting its role as a potential trophic factor for HD. We and others have characterized GDNF's protective effects in neurotoxic rat models of HD (22, 26–30). However, before being tested in patients, GDNF's ability to provide neuroprotection in the HD transgenic mouse should be demonstrated, because this model more closely mimics the genetic nature of human HD.

In the present study we tested the hypothesis that delivery of GDNF into the striatum by a recombinant adenoassociated viral vector (rAAV) can preserve motor function and prevent striatal cell loss in the N171-82Q transgenic mouse model (3). N171-82Q mice contain a human cDNA encoding for the N-terminal fragment of huntingtin with 82 glutamine repeats. This is a shorter number of glutamine repeats compared with those of other transgenic and knockin mouse models (31, 32) and may be more clinically relevant to the number of repeats found in HD patients. Moreover, the shorter repeat length leads to a protracted course of disease compared with what has been observed in the other transgenic mouse models, allowing a potentially larger therapeutic window in which to administer trophic factors such as GDNF. N171-82Q mice exhibit evidence of degenerating neurons, astrogliosis, and the formation of inclusions in the striatum along with motor deficits including loss of coordination, gait abnormalities, hypokinesia, hind limb clasping behavior, and muscle weakness (3, 33). Here we report that bilateral AAV-GDNF delivery provides neuroprotection in the N171-82Q mouse model of HD by enhancing rotorod performance, diminishing hind limb clasping, reducing the density of mutant huntingtin-containing inclusions, and preventing the death and atrophy of striatal neurons.

Results

GDNF and eGFP Expression After rAAV Delivery. Eleven weeks after injection, numerous GDNF or eGFP immunoreactive (ir) cells were seen bilaterally in the striatum in all mice receiving

Conflict of interest statement: M.G., R.T.B., C.D.H., E.P.B., and J.H.K. have a financial interest in Ceregene, Inc.

This paper was submitted directly (Track II) to the PNAS office.

Abbreviations: AAV, adenoassociated viral vector; HD, Huntington's disease; GDNF, glial cell line-derived neurotrophic factor; TH, tyrosine hydroxylase; ir, immunoreactive; mHtt+, mutant huntingtin-positive.

§To whom correspondence should be addressed. E-mail: jkordowe@rush.edu.

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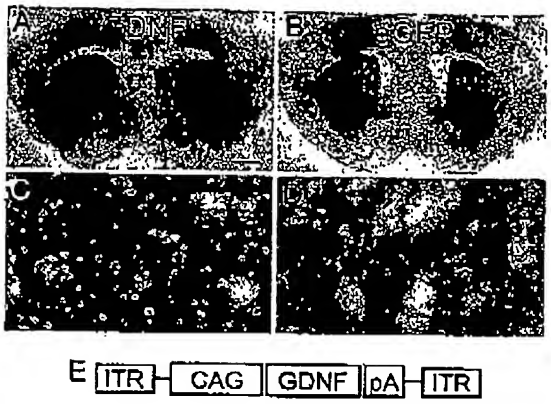

Fig. 1. AAV2 delivers widespread expression of GDNF and eGFP throughout the striatum. Robust GDNF (*A* and *C*) and eGFP (*B* and *D*) immunoreactivity was seen in the striatum of N171-82Q transgenic mice 11 weeks after injection (week 16 of life). High-power magnification (*C* and *D*) shows positive staining in the cell bodies as well as the neurites. GDNF immunoreactivity was never detected in striatal tissue from AAV-eGFP-injected transgenic mice or WT littermates (data not shown). (*E*) Schematic of the AAV-GDNF viral vector genome. (Scale bar: *A* and *B*, 400 μm; *C* and *D*, 16.5 μm.)

AAV-GDNF or AAV-eGFP, respectively (Fig. 1 *A* and *B*). Qualitative observations revealed that GDNF immunoreactivity covered approximately >90% of the striatum and eGFP-ir in >75% of the striatum; this discrepancy may be because GDNF is a secreted protein and eGFP is not. Both cell bodies and fibers coursing through the striatal gray matter stained positive for the appropriate transgene (Fig. 1 *C* and *D*). GDNF and eGFP immunoreactivity was also observed in the overlying cortex as a result of injection spread. Retrograde transport of both GDNF and eGFP was seen in the substantia nigra pars compacta, and anterograde transport was seen in the globus pallidus and substantia nigra pars reticulata (data not shown).

Striatal Delivery of AAV-GDNF Improves Behavioral Function in HD Mice. The accelerating rotorod and hind limb clasping tests evaluated the development of behavioral deficits over the 11 weeks of the study. AAV-eGFP-injected mice performed significantly worse over the course of the study compared with WT littermates ($P < 0.05$, Fig. 2*A*). This effect emerged as statistically significant at week 10 of life. Importantly, AAV-GDNF-treated mice performed significantly better than the AAV-eGFP-injected mice from week 10 to week 16 ($P < 0.05$ for all weeks analyzed). AAV-GDNF-treated mice performed similarly to WT mice until the last 3 weeks of testing ($P < 0.05$).

Mice were evaluated twice a week on the hind limb clasping test. Clasping emerged at week 12 for AAV-eGFP-injected mice, with greater numbers of these mice exhibiting this behavior as the experiment progressed. AAV-GDNF treatment delayed the emergence of clasping, and fewer AAV-GDNF-treated mice clasped at each time point compared with AAV-eGFP-treated mice (Fig. 2*B*). The total number of clasping events per mouse over the duration of the experiment was summed, and significant differences between transgenic groups were observed. ($P < 0.05$) (Fig. 2*C*). WT mice never exhibited clasping behavior.

AAV-GDNF Treatment Prevents Neuronal Atrophy and Death in the Striatum. Upon gross examination, brains from both groups of transgenic mice (AAV-GDNF- and AAV-eGFP-injected) appeared smaller in size compared with those of WT controls. Both AAV-GDNF-injected (12.3 ± 0.4) and AAV-eGFP-injected

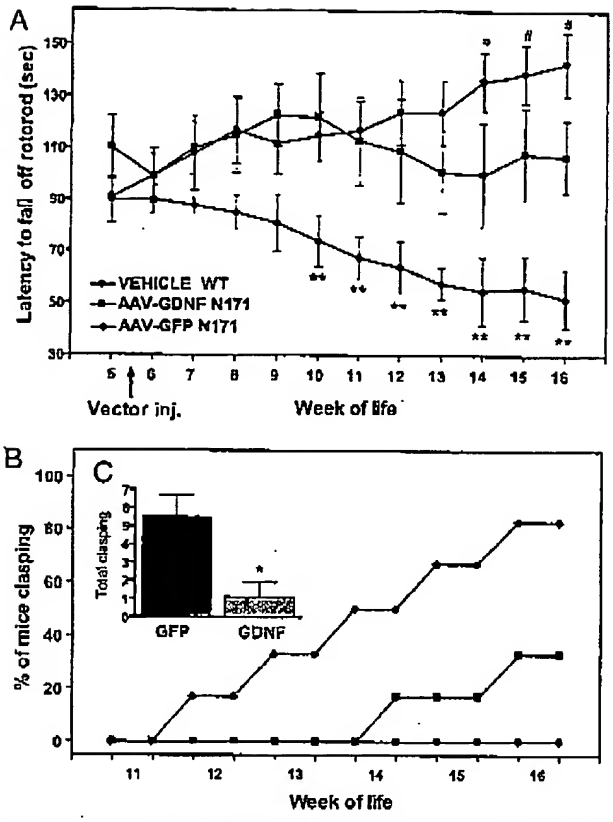

Fig. 2. AAV-GDNF administration attenuates behavioral deficits in N171-82Q transgenic mice. (*A*) AAV-eGFP-injected N171-82Q mice ($n = 6$) performed significantly worse over the course of the study compared with WT ($n = 7$) on the rotorod (**, $P < 0.05$). AAV-GDNF-injected mice ($n = 7$) performed significantly better than AAV-eGFP-injected mice (**, $P < 0.05$) and were only significantly different from WT at the last three time points measured (#). (*B*) Clasping behavior emerged later in life in AAV-GDNF-treated mice compared with AAV-eGFP controls. (*C*) Fewer GDNF-treated mice clasped throughout the study compared with eGFP-treated mice (*, $P < 0.05$).

(11.6 ± 0.9) mice had smaller striatal volumes compared with WT cohorts (15.0 ± 0.3) ($P < 0.001$) (Fig. 3*A*).

Although AAV-GDNF treatment did not prevent the reduction in striatal volume engendered by the HD mutation, it significantly preserved the number and volume of striatal neurons. Unbiased stereological counts estimated $2.9 \times 10^6 \pm 1.2 \times 10^5$ NeuN-positive cells in the striatum of WT mice. Significantly fewer (24%) striatal NeuN-ir neurons were estimated in AAV-eGFP-treated HD mice ($2.2 \times 10^6 \pm 1.7 \times 10^5$) ($P < 0.05$). In contrast, AAV-GDNF-treated mice had significantly more (19%) NeuN-positive striatal neurons ($2.7 \times 10^6 \pm 1.2 \times 10^5$) ($P < 0.05$) compared with AAV-eGFP-treated mice, and estimated counts were statistically similar to WT controls ($P = 0.27$).

In addition to having more striatal NeuN-positive neurons, mice injected with AAV-GDNF also had larger NeuN-positive neurons. The nucleator method was used to quantify the average volume of neuronal cell bodies in the striatum. The mean volume (cubic micrometers) of NeuN-ir striatal neurons from AAV-eGFP-injected mice (502 ± 36.6) was significantly less (20%) than those of WT littermate mice (630 ± 17.3) ($P < 0.01$). NeuN-positive cell bodies in the AAV-GDNF-treated mice (591 ± 26.0) were significantly larger (15%) than those mea-

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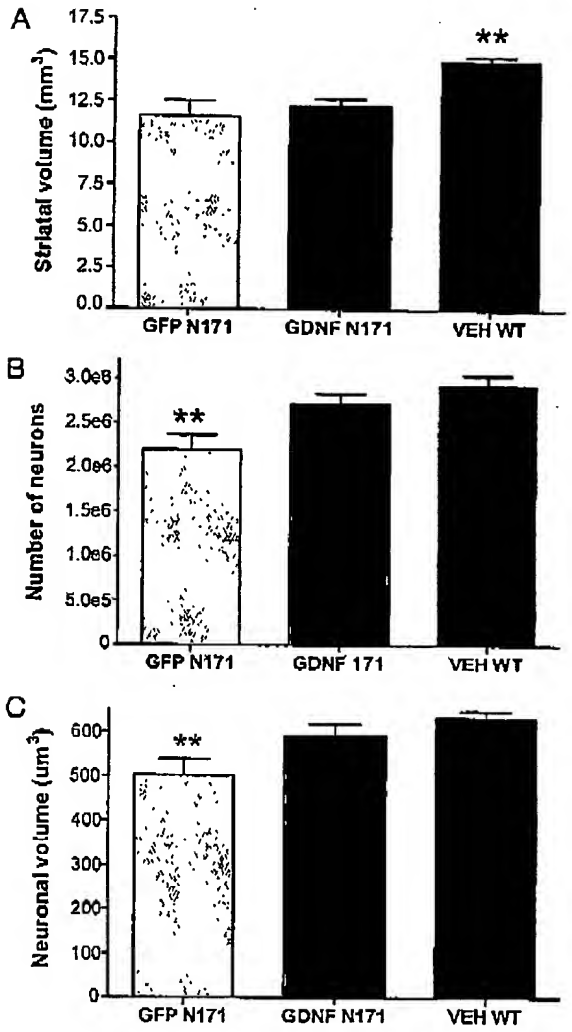

Fig. 3. AAV-GDNF treatment prevents striatal cell death and cell atrophy. (*A*) Both AAV-GDNF-injected mice (18% decrease) and AAV-GFP-injected mice (23% decrease) had significantly smaller striata compared with WT littermates ($P < 0.01$). (*B*) Stereological counts of striatal NeuN-ir neurons demonstrated that AAV-GDNF-treated mice had significantly more striatal neurons compared with AAV-eGFP-treated mice ($P < 0.05$). (*C*) GDNF treatment also prevented neuronal atrophy. AAV-GDNF-injected mice had 15% larger cell bodies compared with AAV-eGFP-injected mice. Striatal volume, neuronal number, and neuronal size analyses were performed at week 16 of life.

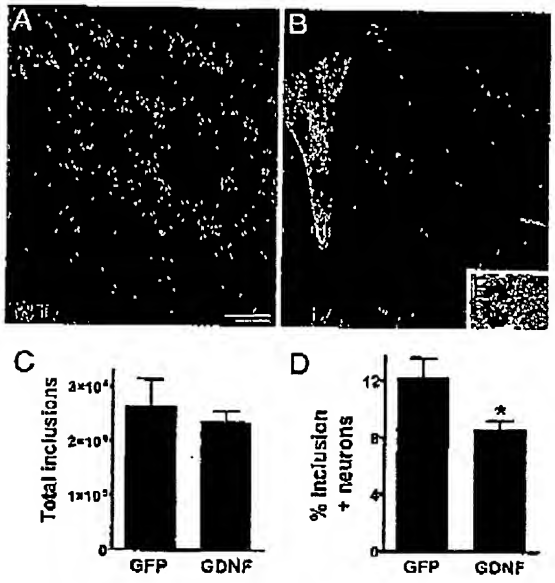

Fig. 4. AAV-GDNF reduces the percentage of neurons with mHtt+ inclusions. WT mice never exhibited mHtt+ inclusions in the striatum (*A*). In contrast, transgenic mice, regardless of group (the example above is an AAV-eGFP-injected mouse), showed evidence of mHtt+ inclusions in the striatum (*B*). (*E*) Evidence of cells with robust inclusions. (*C*) Whereas no differences in the total number of striatal inclusions were observed between AAV-GDNF-injected mice and AAV-eGFP-injected mice ($P > 0.05$), a significant decrease in the percentage of neurons with inclusions was observed (*D*) ($P < 0.01$). (Scale bar: *A* and *B*, 500 μm; *E*, 10 μm.) Inclusion quantification was performed at week 16 of life.

sured from AAV-eGFP-treated mice ($P < 0.05$) and statistically similar to WT controls ($P > 0.05$).

In addition to stereological estimation of the number and size of striatal neurons, we evaluated GDNF's potential effects on the nigrostriatal dopamine system by measuring the optical density of tyrosine hydroxylase (TH)-positive fibers in the striatum. Average values for striatal optical density expressed as mean ± SEM were as follows: AAV-eGFP, 140 ± 6.6; AAV-GDNF, 160 ± 7.3; WT, 150 ± 4.2. There were no statistical differences in TH optical density values among the three groups ($P = 0.26$).

AAV-GDNF Alters Mutant Huntingtin Pathology in HD Mice. Mutant huntingtin-positive (mHtt+) inclusion bodies are a prominent pathological feature in HD patients that are replicated in most transgenic HD models. mHtt+ inclusions were never present in WT mice (Fig. 4*A*) but were present in N171-82Q transgenic mice, regardless of treatment group (Fig. 4*B*) and characterized by dark, dense inclusion bodies (*E*). Stereological counts of EM48-ir striatal inclusions demonstrated no significant differences between mice injected with AAV-GDNF (235,710 ± 20,159) and mice injected with AAV-eGFP (264,621 ± 49,612) (Fig. 4*C*) ($P > 0.05$). However, because there were significantly more NeuN-ir neurons in the striata of AAV-GDNF-injected mice, we also evaluated the ratio of neurons in the striatum that contained inclusion bodies. Mice treated with AAV-GDNF had a significantly lower percentage (8.6 ± 0.03%) of striatal neurons that contained EM48-ir inclusions compared with AAV-GFP-treated mice (12.2 ± 1.4%) (Fig. 4*D*) ($P < 0.01$).

Discussion

To our knowledge, this study is the first to demonstrate that viral delivery of GDNF to the striatum of presymptomatic mice can prevent the structural and functional degeneration seen in a transgenic model of HD. We observed robust gene expression of GDNF and eGFP for up to 11 weeks after viral delivery to the striatum. AAV-GDNF improved rotorod performance, delayed and attenuated clasping behavior, and preserved the number and size of striatal neurons in this model. Both clinical (34–36) and preclinical (3, 31, 33) studies suggest that HD symptoms can occur before the frank loss of striatal neurons. The prevention of neuronal atrophy by AAV-GDNF suggests that an early aspect of the degenerative process that mediates symptom onset might be prevented by this treatment. The lack of difference in whole striatal volume of AAV-GDNF-treated mice compared with AAV-eGFP-treated mice suggests potential atrophy of neuronal processes within the striatum, either those that arise

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from striatal neurons themselves or those that course through the striatum via the internal capsule. Interestingly, the protection of striatal neurons was associated with a lower percentage of neurons containing inclusion bodies consisting of pathogenic, mutated huntingtin protein. It is unknown whether GDNF partially prevented the formation of inclusion bodies or assisted in their breakdown and subsequent processing by the ubiquitin proteosome pathway. Alternatively, GDNF may have been capable only of protecting cells that had not yet formed inclusions. Empirical studies should address this question, the answer to which will be important in the timing of AAV-GDNF administration, especially if the latter is the case and GDNF is unable to elicit trophic effects on cells that have already formed inclusion bodies. These data extend previous demonstrations by our laboratory and others (22, 26–30) that AAV-GDNF is protective in neurotoxin-based HD models and now establish its beneficial effects in a transgenic mouse model.

The data presented in this study stand in contrast to the study by Popovic *et al.* (37), who delivered GDNF via a lentiviral vector into the striata of R6/2 transgenic HD mice. Although excellent gene expression was demonstrated in the striatum, they did not observe functional improvement or differences in the number of mHtt+ striatal inclusions compared with control mice. This difference may be attributable to the transgenic mouse model used. R6/2 mice contain a human cDNA that encodes mutant huntingtin protein with 145 CAG repeats, the larger number of repeats resulting in a more severe model in terms of the time course of behavioral and pathological sequelae compared with N171-82Q mice. In their study, R6/2 mice were treated with lentiviral GDNF at week 5, when the anatomical and behavioral symptoms had potentially already commenced. Recent reports demonstrate that inclusions have already formed in the striatum of R6/2 mice by postnatal day 1, brain weight is significantly reduced by 4 weeks of age (38), and by week 5 R6/2 mice show significant reductions in running, climbing, and open-field behavior compared with WT littermates (39). In the present study, AAV-GDNF was administered to the N171-82Q mice at week 5, and GDNF was already optimally expressed before the behavioral syndrome began, around week 10. Taken together, these two studies suggest that gene delivery of GDNF may be beneficial when applied to asymptomatic HD gene carriers but may be less effective for patients already displaying behavioral symptoms. If further studies validate this hypothesis, testing surgical treatment strategies in asymptomatic patients will be a future challenge given the number of patients required and the time needed to ascertain a clinical readout.

GDNF's ability to rescue dopaminergic neuron loss in patients with Parkinson's disease has been evaluated in three published clinical trials to date (40–42). Although the efficacy of GDNF to improve motor scores on the Unified Parkinson's Disease Rating Scale has been variable, reported side effects in these studies were mostly attributed to problems with the delivery methods used, including putamenal delivery via a pump (41, 42) and intracerebroventricular delivery (40). Side effects that were most likely due to GDNF itself were minimal and included nausea, vomiting, headache, and L'Hermitte's sign (40–42).

The exact mechanism of how mutant huntingtin leads to the devastating course of events that culminates in cell death in HD remains elusive. It has been suggested that mutant huntingtin is cleaved and translocates into the nucleus, where it activates apoptosis (43). Additionally, it has been demonstrated that the lack of normal huntingtin in HD patients and transgenic mice leads to a decrease in the transcription of trophic factors, such as BDNF (44). Upon binding to its GFRα1/Ret receptor complex, GDNF activates the inositol triphosphate and the mitogen-activated protein kinase intracellular cascades. Stimulation of these pathways results in cell and neurite outgrowth and inactivation of caspases 3 and 9 (via Akt), effectively inhibiting apoptosis. Although GDNF's exact role in preventing cell death in the N171-82Q transgenic model of HD remains to be established, we speculate that increasing trophic support and inhibiting apoptosis via these two pathways likely play integral roles.

The present study demonstrates that the viral delivery of GDNF protects striatal neurons from mutant huntingtin-induced cell death. Additionally, AAV-GDNF treatment significantly attenuates impairments with balance and coordination and delays the hind limb clasping phenomenon. These results support the concept that the striatal delivery of AAV-GDNF may be a viable therapy for patients with HD.

Materials and Methods

Animals. N171-82Q transgenic mice [strain B6C3F1/J-TgN(HD82Gln)81Dbo] and WT littermates (B6C3F1/J background strain) were used in this experiment. Breeding pairs were obtained from The Jackson Laboratory, and all mating took place at Rush University. Male N171-82Q transgenic mice were always bred with female WT littermates. Mice were housed in groups of either two or three per cage on a 12-h light/dark cycle, with chow and water provided ad libitum. For all analyses, the same three groups of mice were used: group 1, vehicle-injected WT mice ($n = 7$); group 2: AAV-GDNF-injected N171-82Q mice ($n = 7$); group 3, AAV-eGFP-injected N171-82Q mice ($n = 6$). All experiments were carried out in accordance with federal guidelines of proper animal care and with the approval of the Rush University Medical Center Animal Care Committee. PCR was performed to genotype all mice by using primers previously described (3). CAG repeat number was confirmed by a separate PCR assay with primers flanking the repeat sequence, followed by polyacrylamide gel electrophoresis of products and comparison of bands with known size standards from human HD patients.

Construction of AAVs. AAV-GDNF and AAV-eGFP viral vectors (serotype 2) were used in this study. The vector genome consisted of the GDNF or eGFP expression cassette flanked by the inverted terminal repeats from AAV2. The expression cassette consisted of the hybrid CAG promoter (including a human cytomegalovirus enhancer, a chicken β-actin promoter and splice donor, and a rabbit β-globin splice acceptor) driving expression of the GDNF or eGFP cDNAs and the polyadenylation sequence from human growth hormone gene. Viral vectors were produced in human embryonic kidney 293 (HEK293) cells by using the calcium phosphate triple plasmid transfection method. Three days after transfection cells were harvested and lysed. AAV was purified from the cell lysates by heparin and ion-exchange column chromatography. Purified particles were concentrated by centrifugal filtration, and vector titer was determined by quantitative PCR. All vectors were created by Ceregene.

rAAV Injections. At 5 weeks of life, mice were anesthetized with a ketamine (10 mg/kg) and xylazine (100 mg/kg) mix (0.1 ml per 10 g per mouse, administered i.p.), and their heads were shaved, sterilized with betadine, and placed in a mouse stereotaxic frame (Kopf Instruments, Tujunga, CA). A midline incision was made, and bilateral burr holes were created over the striatum with a dental drill. One injection (2 μl) of AAV-GDNF, AAV-eGFP, or vehicle was made on each side of the striatum (0.86 mm rostral to bregma, 1.8 mm lateral to midline, and 3.5 mm ventral to the skull surface). Each mouse received a total of 4×10^9 vector genomes, distributed bilaterally. All injections were performed through a 10-μl Hamilton syringe connected to an infusion pump at a rate of 0.2 μl/min. Needles (33-gauge, blunt-tipped) were left *in situ* for an additional 5 min to allow the injectate to diffuse

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from the needle tip. The scalp was closed with 5-0 polyvicryl suture.

Behavioral Analyses. All behavioral analyses were performed in a blinded fashion.

Accelerating Rotorod Test. All mice were assessed (twice per week) on the accelerating rotorod test beginning at week 4 of life (1 week before injection of viral vectors) and every week thereafter until they were killed at postnatal week 16. The rod accelerated from 0 to 30 rotations per minute over 300 s, and the latency to fall was recorded. Three trials were performed in each testing day, with a 1-h interval between trials. At each time point and for each animal, the three trials latency-to-fall were recorded, and the average was used for statistical analysis.

Hind Limb Clasping Test. All mice were assessed (twice per week) via the clasping test beginning at week 4 of life and every week thereafter until they were killed. The clasping test evaluated the mice's hind limb response during tail suspension 10 cm above their home cage. Mice received a score of 0 for a normal hind limb extension and a score of 1 when hind limbs were clasped.

Immunohistochemical Analysis. By using the biotin-labeled antibody procedure described previously (30), 40-μm-thick, free-floating, coronal sections were submitted to a series of protein detection analyses using antibodies against NeuN (1:1,000; Chemicon), GDNF (1:250; R & D Systems), eGFP (1:2,000; Clontech), TH (1:10,000; Chemicon), and mutant huntingtin (mEM48 clone, 1:50; Chemicon). Briefly, primary antibody incubations (overnight) were followed by secondary antibody incubations (1 h) with the appropriate biotinylated IgG secondary antibodies (1:200; Vector Laboratories). Nickel intensification was used in the GDNF staining procedure. Controls consisted of substitution of an irrelevant IgG in lieu of the primary antibody.

Stereological Analysis. Neuronal counts of NeuN-ir or mutant huntingtin-ir neurons were performed by using an unbiased, design-based stereology procedure as described previously (30). Neuronal and inclusion counts for each subject were made throughout the striatum by using five equally spaced serial sections spaced 480 μm apart. Using STEREOINVESTIGATOR software (Microbrightfield), the optical fractionator method estimated the number, and the nucleator procedure quantified the cell volume of NeuN-ir cells. The optical fractionator method was also used to estimate the number of mHtt+ inclusions. Striatal volume was quantified on the same five equally spaced sections of NeuN-stained tissue by using the Cavalieri method.

TH Optical Density Analysis. The striatum was outlined at ×2 magnification and the optical density of TH-positive fibers was assessed by using the SCION image analysis program. Background levels were captured from the corpus callosum in each section and subtracted from the total optical density measurement. The optical density number reported is the average optical density from each of the five measured sections. All analyses were performed in a blinded fashion.

Statistical Analysis. A two-way repeated-measures ANOVA with Student–Neuman–Keul's post hoc analyses was used to compare group performance on the rotorod test. Hind limb clasping behavior was summed for each animal across time, and Kruskal–Wallis nonparametric analyses, along with Mann–Whitney pairwise comparisons, assessed significant differences between individual groups. The number and size of NeuN-ir cells in the striatum, the number of inclusions, total striatal volume, and TH optical density were compared by using a one-way ANOVA with Scheffé's post hoc tests to assess for significant differences between individual groups.

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